

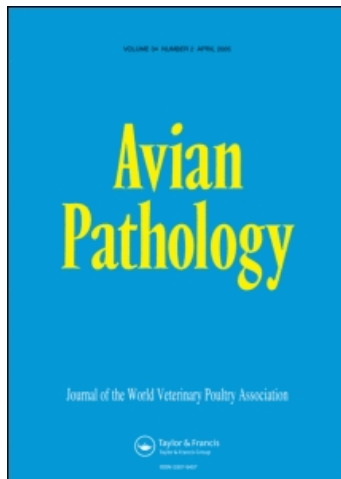
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# Occurrence of avian leukosis virus subgroup J in commercial layer flocks in China

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Mortality from myeloid leukosis was observed in commercial layers from 12 farms in northern China. Affected chickens were extremely thin and dehydrated, bleeding occurred in feather follicles and claws, combs were pale and anaemic, phalanges were swollen, and many yellowish–white tumours were seen on the visceral surface of the sternum. Focal tumour cells, with spherical eosinophilic granules in the cytoplasm, were found in the liver, spleen, kidney, ovary, oviduct, lung, bone marrow, proventriculus and gut by histopathological examination. Immunohistochemical studies with a monoclonal antibody to gp85 of avian leukosis virus subgroup J (ALV-J) revealed antigen in all organs examined. Polymerase chain reaction tests using a pair of ALV-J-specific primers H5/H7 (Smith *et al.*, 1998) produced a 545 basepair fragment. The sequence of the Polymerase chain reaction product was compared with that of the ALV-J HPRS-103 prototype strain. The identity of nucleotides and predicted amino acids was 97.4% and 96.1%, respectively. On this basis the disease in the egg-type chickens was diagnosed as an ALV-J infection. This is the first report of field cases of myeloid leukosis caused by ALV-J in commercial egg-type chickens.

## Introduction

Since the first report of myeloid leukosis (ML) caused by the subgroup J avian leukosis virus (ALV-J) in Britain (Payne *et al.*, 1991), it has occurred in broiler breeder flocks in many countries causing serious losses. The virus spreads horizontally and vertically, and has brought increasingly severe damage to the poultry industry throughout the world. The fact that ALV-J is apparently a recombination between endogenous viral genes and other ALVs has caused concern about the emergence of new recombinants and subgroups of ALV (Van der Heide, 1998). In recent years, studies have shown that sequence changes of *env* genes in the variable regions cause rapid evolution and variation of antigenicity of ALV-J (Venugopal *et al.*, 1998). Although egg-type (Leghorn) chickens are susceptible experimentally to

induction of ML and other tumours by ALV-J, naturally infected commercial egg-type chickens have not been found until recently anywhere in the world.

In 2002, ML was found in egg-type chickens in northern China. In order to determine the nature of the disease, the pathology, viral immunohistochemistry, and sequence analysis of the Polymerase chain reaction (PCR) product amplified from the associated ALV were studied.

## Materials and Methods

### Observations on the affected flocks

Affected chickens of a commercial strain of brown egg-layers came from 12 farms in northern China. One farm was also a breeder farm. Flock sizes ranged from 6000 to 25000 chickens, and these were fed with mixed feed in cages in one-storey houses. The chickens were extremely thin with anaemia and dehydration, and many died. Production

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performance of the egg-layers decreased to 70%, and 12 farms were affected over the same period.

### Autopsy

Eighteen dead chickens and 32 chickens with extreme emaciation, anaemia and haemorrhages in feather follicles were submitted to the laboratory by the owner for necropsy. Four affected hens were selected from each of the 12 farms, together with two cocks from the breeder farm. Four batches of chickens (50 in total) were necropsied, respectively, at 70 days of age (two cocks), 150 days (eight hens), 170 days (36 hens), and 260 days (four hens).

### Histopathology

Tissues examined included the bone marrow, liver, kidney, spleen, lung, heart, pancreas, oviduct, ovary, proventriculus, intestine, skeletal muscle, cerebrum, testes, and sciatic nerve. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 µm, and stained with haematoxylin and eosin.

### Immunohistochemistry

Tissues for immunohistochemical examination came from 30 chickens, two or three from each farm, diagnosed with ML by histopathological examination. Two types of control tissues were developed. In control class 1 the materials were identical to those of the ML class; but the ALV-J monoclonal antibody was replaced by a monoclonal antibody against Newcastle disease virus (kindly provided by Youquan Cheng, Institute of Farming and Veterinary of Fujian Agricultural Science College). The tissues of control class 2 came from negative specific pathogen free chickens obtained from the Research Center of Experimental Animals in Beijing. Immunohistochemical examination was carried out as described by Wang *et al.* (2001). Briefly the tissues were deparaffinized in xylene, transferred to pure alcohol, and passed through a graded alcohol series to water. Sections were treated in 3% hydrogen peroxide for 20 min, then blocked with normal sheep serum for 20 min. Sections were sequentially incubated for 1 h at 37°C with monoclonal antibody F144 against ALV-J gp85 (Qin *et al.*, 2001) provided by the USDA Avian Disease and Oncology Laboratory, (East Lansing, MI, USA) and sheep anti-mouse immunoglobulinG labelled with horseradish peroxidase (from Beijing Dingguo Biotechnology Development Center). Tissues were rinsed with phosphate-buffered saline three times, for 5 min at each time, and between each treatment step, except for the blocking step with normal sheep serum, which was not rinsed. Sections were developed for 7 min in 0.05 M diaminobenzidine with 1% hydrogen peroxide, and were washed with distilled water to stop the reaction. Tissues were counterstained for 1 min in haematoxylin. Sections were treated for 10 sec in alcohol with 1% hydrochloric acid, and then washed for 10 min in running water. They were dehydrated in a graded alcohol series, mounted and examined.

### Polymerase chain reaction

PCR with primers H5/H7 was used to test total DNA from frozen (–80°C) tumour, bone marrow, oviduct, liver, and spleen from the dead and sick chickens from seven farms for the presence of sequences specific for ALV-J as described (Smith *et al.*, 1998). The PCR mixture consisted of the following in a total volume of 50 µl: double-distilled water (40.5 µl), dNTP each at 200 mM (1 µl), 10 × buffer (5 µl), H5 and H7 at 20 pmol/µl (each 1 µl), 5 U Taq DNA polymerase (0.5 µl) (TAKARA Bio Inc., Dalian, China), and template DNA (1 µl). At the same time, template DNA from specific pathogen free chickens was used as negative controls. PCR programme consisting of the following steps was performed for the amplification: denaturation at 93°C for 1 min, annealing at 60°C for 1 min decreasing by 2°C in each cycle, and extension at 72°C for 90 sec for seven cycles, followed by 30 cycles of 93°C for 1 min, 48°C for 1 min, 72°C for 90 sec, with a final extension at 72°C for 10 min. Five microlitres of the amplified products were mixed with 2 µl gel loading buffer, and examined by 1% agarose gel electrophoresis in 1 × TAE buffer (pH 8.0), for 40 min at 50 V.

### Sequencing and comparison of the PCR amplified product

The PCR product was sequenced by TAKARA Bio Inc. The sequence (GenBank accession number AY360088) was compared with that of the ALV-J prototype HPRS-103 strain, and identities of predicted encoded amino acid sequence were calculated.

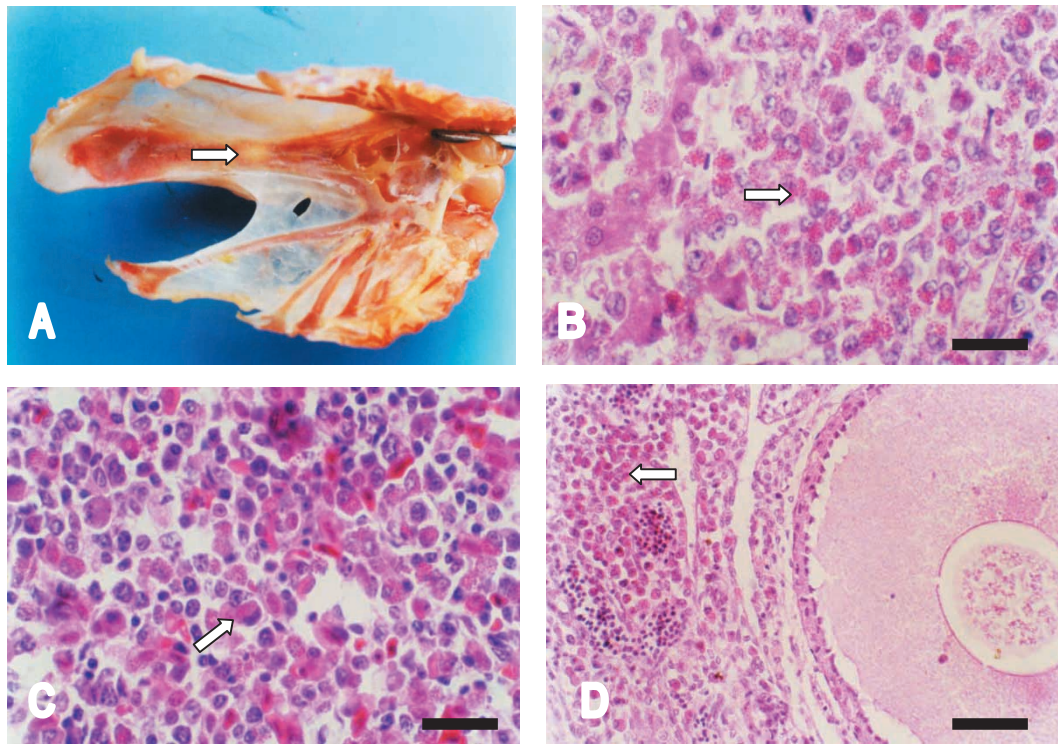
## Results

### Clinical signs and gross lesions

Clinical signs shown by birds from the 12 farms included depression, loss of appetite, extreme emaciation, a pale comb with marked anaemia, and haemorrhages in the skin of phalanges and feather follicles. The mortality rate was over 10%. The yellowish–white tumours were observed in the phalanges and on the visceral surface of the sternum (Figure 1a), in nodular formations. The surface of some tumours had a layer of thin and brittle periosteum. Most tumours in phalanges grew on one side. The colour of the bone marrow depended on the course of the disease; the colour was pale in the late stage. Livers were only slightly swollen with mottled grey–white pinpoint spots under the capsule. Spleens were enlarged slightly to several times and had yellowish–white tumour nodules in a few cases. Kidneys were markedly swollen, and some had light grey–white mottled tumour masses. Ovaries and oviducts of 15/36 hens were undeveloped at 170 days of age. The testes of one cock were poorly developed at 70 days of age and the size was only one-eighth of the normal at that age. A few proventriculi were swollen. The proventricular papillae were flat in 25/50 cases. No gross lesions were observed in the heart, lung, pancreas, or cerebrum.

### Histopathology

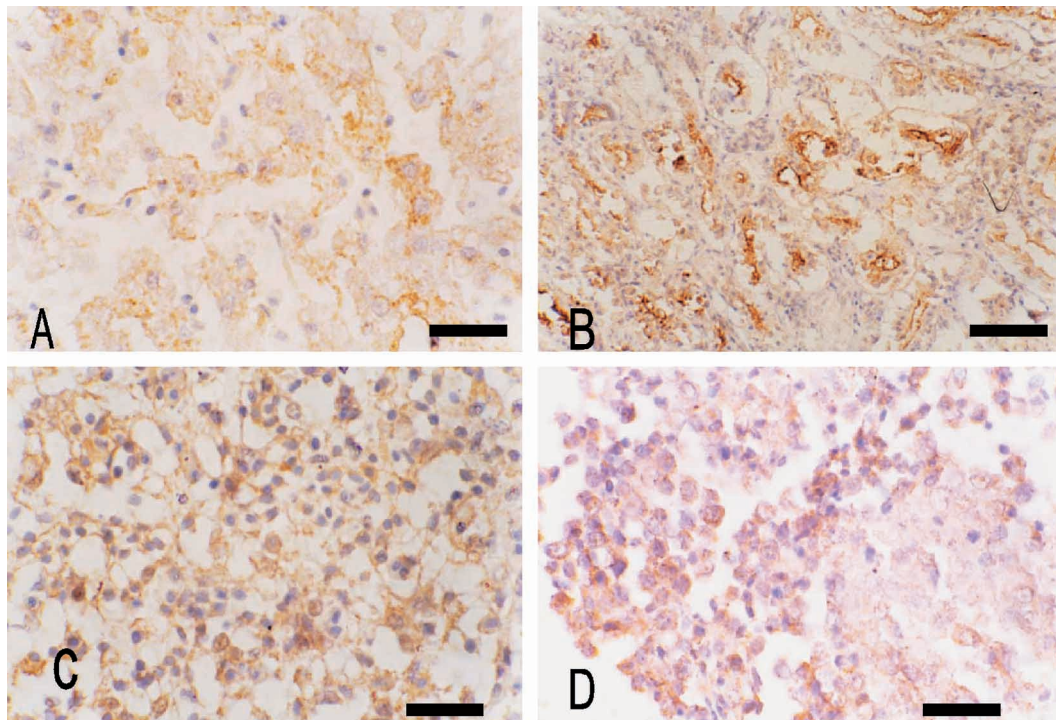
Microscopically, 45/50 birds had ML; no other types of tumour were seen. The other five birds had no ML tumours and were not examined further. Tumours in bone were composed of uniform ML cells similar in appearance to normal myeloid cells. The nuclei often lay to one side of the cell and had a marked nucleolus. Cytoplasm was filled with conspicuous spherical eosinophilic granules. Many proliferated focal tumour cells destroyed the normal structure of the bone marrow. In the liver, the spaces of Disse were expanded by serous material. Hepatocytes were atrophied and thin. Tumour cells were present around veins and arteries in the liver and grew focally (Figure 1b). In the spleen, lymphocytes decreased and tumour cells were widely present in both the red pulp and the white pulp (Figure 1c). Tumour cells in the lungs were observed in the pneumocapillares and parabronchial lobules. Epithelial cells of renal tubules were swollen, degenerate and separated from the basement membrane. Tumour cells in the interstitium grew focally. Tumour cells were present in the



**Figure 1.** Gross and histopathological lesions (haematoxylin and eosin staining) in naturally diseased ALV-J-infected egg-type chickens. (a) Inside of the sternum showing nodular tumours. The arrow indicates yellowish-white tumour on the visceral surface of the sternum. (b) Liver including myeloid tumour cells. The arrow indicates myeloid tumour cells. Bar = 20  $\mu$ m. (c) Spleen showing myeloid tumour cells. The arrow indicates myeloid tumour cells. Bar = 20  $\mu$ m. (d) Ovary showing myeloid tumour cells. The arrow indicates myeloid tumour cells. Bar = 50  $\mu$ m.

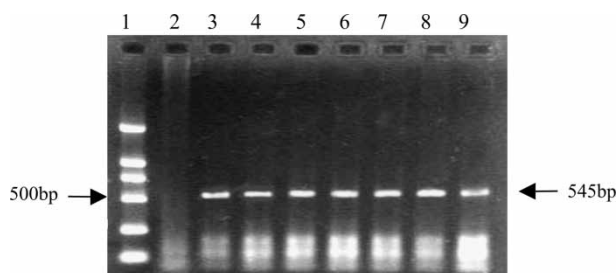
mucous membrane of the proventriculus. There were tumour cells in the lamina propria of the intestinal mucosa. Pancreatic gland alveoli were

degenerated and necrotic. Many tumour cells gathered around blood vessels. Proliferated tumour cells grew focally in the ovary and oviduct (Figure



**Figure 2.** Photomicrographs of immunohistochemical staining for ALV-J gp85 antigen in tissues from ALV-J naturally diseased egg-type chickens. (a) Positive staining in the liver, 70-day-old hen. Bar = 20  $\mu$ m. (b) Intense staining in the free surface of the renal tubule, 150-day-old hen. Bar = 50  $\mu$ m. (c) Positive staining in the reticular cells and macrophages of the spleen, 260-day-old hen. Bar = 20  $\mu$ m. (d) Positive staining in the myeloid tumour cells of the bone marrow, 170-day-old hen. Bar = 20  $\mu$ m.





**Figure 3.** PCR amplification of DNA obtained from multiple tissues of egg-type chickens in different farms. Lane 1, DNA molecular weight marker DL2000. Lane 2, Negative control with no template. Lane 3, Tumour from farm 1. Lane 4, Liver from farm 2. Lane 5, Oviduct from farm 3. Lane 6, Bone marrow from farm 4. Lane 7, Spleen from farm 5. Lane 8, Liver from farm 6. Lane 9, Tumour from farm 7.

1d). Few myeloid tumours were observed in the epicardium, and no tumour cells were seen in the testes, sciatic nerve, or skeletal muscle.

### Immunohistochemistry

In the ML affected group, all samples of each of 30 chickens were positive for ALV-J gp85. Positive staining was observed in the liver (Figure 2a), the kidney (Figure 2b), the heart and spleen (Figure 2c), the pancreas, brain, oviduct, lung, ovary, and proventriculus. Myeloid tumour cells in the bone marrow were also stained (Figure 2d). There was no staining in the two control classes.

### Sequence analysis of PCR product and predicted amino acids.

Fragments of about 545 kb were obtained separately by PCR using total DNA from the tumour, bone marrow, oviduct, spleen, and liver from seven of the 12 farms. All tissues examined by PCR were positive for ML and for ALV-J gp85 by immunohistochemistry. The PCR products were consistent in size with the predicted fragment (Figure 3). Sequence analysis of the fragment of partial gp85 *env* gene and the 3' region of the *pol* gene obtained from the infected egg-type chickens showed that there were only 14 base pairs different from that of the ALV-J prototype HPRS-103 strain; their identities were nearly 97.4% (GenBank accession number AY360088). The identities of predicted amino acid sequences between this PCR amplified product and that of the ALV-J prototype HPRS-103 strain was nearly 97.1%; there were only seven amino acids that had changed.

### Discussion

Currently, reports on the global prevalence of ALV-J are limited to meat-type chickens. In this present study, the occurrence of ML in commercial brown layer flocks is reported. This is the first report of naturally occurring ML caused by ALV-J in

commercial layer stock. However, Gingerich *et al.* (2002) reported ML in commercial White Leghorn flocks used to produce eggs for human vaccine production from which a recombinant ALV containing the long terminal repeat (LTR) of subgroup J and the envelope of subgroup B ALV was isolated.

The disease shown in the brown layers was initially diagnosed as ML with a possible aetiology of ALV-J based on the epidemiology, clinical signs, pathological signs at autopsy, histopathology, and the shape and structural character of myeloid tumour cells (Yin *et al.*, 1997). This was confirmed by detecting the specific ALV-J antigen by the immunohistochemical assay using a monoclonal antibody against the ALV-J gp85 and by PCR for ALV-J and sequence analysis of the PCR product. Since the PCR-positive birds all originated from the same breeder farm, only one PCR product has been sequenced to date. This sequence was very similar to that of the prototype ALV-J strain, HPRS-103.

The chickens on the 12 farms came from the same breeder farm, and breeder chickens submitted for examination from the breeder farm were diagnosed as positive for ML and ALV-J by histopathology, immunohistochemistry and PCR. The affected layer breeder flocks were not reared with meat-type chickens, and the disease on the 12 farms occurred almost concurrently, suggesting that vertical spread of ALV-J from the breeders was the main source of the infection. It is not known how the parent breeding stock became infected with ALV-J. The grandparent stock were reared in China but their infective status is not known.

Ovaries and oviducts of 15/36 hens infected by the virus were undeveloped at 170 days of age, and this directly influenced the production performance of the layers. The disease may become one that gravely endangers the egg poultry industry, and much importance should be attached to it. Avian leukosis can cause significant economic losses because of high mortality, tumour occurrence, and the cost of eradication. In order to eradicate the disease, many meat-type breeder companies have to spend much money and time on virus screening and elimination. The presence of ALV-J in egg-type chickens brings a new challenge to the poultry industry to which poultry veterinarians must pay attention.

### Acknowledgements

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## RÉSUMÉ

### Apparition du sous-groupe J du virus de la leucose aviaire, dans des troupeaux de poules en Chine

Dans 12 élevages de poules commerciales situés au Nord de la Chine, il a été observé de la mortalité due à la leucose myéloïde (ML). Les animaux affectés étaient très maigres et déshydratés, des hémorragies étaient notées au niveau des follicules plumeux et des griffes ; les crêtes étaient très pâles et anémiées, les phalanges étaient gonflées et de nombreuses tumeurs blanc-jaunâtre ont été observées du côté viscéral du sternum. L'examen histopathologique a révélé la présence de cellules tumorales en foyer, avec des granules éosinophiles sphériques dans le cytoplasme du foie, de la rate, des reins, de l'ovaire, de l'oviducte, des poumons, de la moelle osseuse, du proventricule et de l'intestin. Les études immunohistochimiques avec l'anticorps monoclonal anti gp85 du sous-groupe J du virus de la leucose aviaire (ALV-J) a révélé la présence d'antigène dans tous les organes examinés. Les tests PCR utilisant une paire d'amorce spécifique de l'ALV-J H5/H7 (Smith et al. 1998) ont donné un fragment de 545bp. La séquence du produit de la PCR a été comparée à celle de la souche prototype d'ALV-J HPRS-103. L'identité des nucléotides et des acides aminés déduits a été respectivement de 97,4% et de 96,1%. Sur ces bases, la maladie chez les poules a été diagnostiquée comme étant une infection à ALV-J. Cet article est le premier décrivant des cas de leucose myéloïde causés par un ALV-J chez des futures poulettes commerciales de type ponte.

## ZUSAMMENFASSUNG

### Vorkommen von aviärem Leukosevirus Untergruppe J in kommerziellen Legehennenherden in China

In kommerziellen Legehennen aus 12 Farmen in Nordchina wurden Todesfälle durch Myeloidleukose beobachtet. Betroffene Hennen waren extrem abgemagert und dehydriert, an den Federfollikeln und an den Krallen traten Blutungen auf, die Kämme waren blass und anämisch, die Zehen waren geschwollen und auf der visceralen Oberfläche des Sternums waren viele gelblich-weiße Tumore zu sehen. In der histopathologischen Untersuchung wurden Herde von Tumorzellen mit kugelförmigen eosinophilen Granula im Zytoplasma in Leber, Milz, Niere, Eierstock, Eileiter, Lunge, Knochenmark, Drüsenmagen und Darm gefunden. Immunhistochemische Untersuchungen mit einem monoklonalen Antikörper gegen gp85 des aviären Leukosevirus Untergruppe J (ALV-J) ließen Antigen in allen untersuchten Organen erkennen. Die PCR mit einem Paar ALV-J-spezifischer Primer H5/H7 (Smith et al. 1998) produzierte ein 545 bp-Fragment. Die Sequenz des PCR-Produkts wurde mit der des ALV-J HPRS-103-Prototypstamms verglichen. Die Identität der Nukleotide und der vorhergesagten Aminosäuren betrug 97,4 bzw. 96,1 %. Aufgrund dieser Ergebnisse wurde die Erkrankung der Legetiere als ALV-J-Infektion diagnostiziert. Dies ist die Erstbeschreibung von Feldfällen von Myeloidleukose in kommerziellen Hühnern des Legetyps, die durch ALV-J verursacht wurden.

## RESUMEN

### Descripción de un virus de leucosis aviar subgrupo J en una manada de ponedoras comerciales en China

Se observó mortalidad debida a leucosis mieloide (ML) en ponedoras comerciales de 12 granjas en el norte de China. Las aves afectadas estaban extremadamente delgadas y deshidratadas, los folículos de las plumas y las patas presentaban hemorragias, las crestas estaban pálidas y anémicas, las falanges estaban hinchadas y se observaron gran cantidad de tumores blancos-amarillentos en la superficie visceral del esternón. Se describió la presencia focal de células tumorales, con gránulos eosinofílicos esféricos en el citoplasma, en hígado, bazo, riñón, ovario, oviducto, pulmón, médula ósea, proventrículo y intestino en el examen histopatológico. Estudios inmunohistoquímicos con anticuerpos monoclonales frente a la gp85 del virus de leucosis aviar subgrupo J (ALV-J) revelaron la presencia de antígeno en todos los órganos examinados. Las pruebas de PCR en las que se usó un par de cebadores H5/H7 específicos para ALV-J (Smith et al. 1998) amplificaron un fragmento de 545bp. Se comparó la secuencia obtenida a partir del fragmento de PCR con la de la cepa prototipo ALV-J HPRS-103. La similitud nucleotídica y aminoacídica deducida fue del 97,4% y del 96,1% respectivamente. En base a estos datos, la enfermedad en las aves ponedoras se diagnosticó como infección por ALV-J. Esta es la primera descripción de casos de campo de leucosis mieloide causada por ALV-J en ponedoras comerciales.